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(54) Title: VACCINES FOR EQUINE HERPESVIRUS TYPE-1: VIRUSES HARBORING MUTATIONS WITHIN THE IMMEDIATE EARLY GENE

(57) Abstract: This invention relates to mutant Equine herpesvirus type-1 (EHV-1) isolates, and in particular, EHV-1 isolates carrying mutations in the immediate-early (IE) gene of the viral genome. A panel of EHV-1 mutant isolates have been described. Preffered EHV-1 isolates are those which are replication-competent and nonpathogenic. The mutant EHV-1 isolates of the present invention are useful in formulating vaccine compositions for preventing and treating EHV-1 infections in horses. The present invention further provides methods of determining the pathogenicity of an EHV-1 virus present in a horse subject which has been previously immunized with a non-pathogenic EHV-1 isolate of the present invention.

Vaccines For Equine Herpesvirus Type-1: Viruses Harboring Mutations Within the Immediate Early Gene

This invention relates to mutant Equine herpesvirus type-1 (EHV-1) isolates, and in particular, EHV-1 isolates carrying mutations in the sole immediate-early (IE) gene of the viral genome. The present invention further relates to vaccine compositions and methods of treating EHV-1 infections. Methods of determining the pathogenicity of an EHV-1 virus are also provided.

Equine herpesvirus type-1 (EHV-1) is a major pathogen in horses. In infected animals, it is usually associated with upper respiratory tract infections, but may also cause neurological sequelae. EHV-1 infection in pregnant mares causes abortigenic disease and has an important economic impact on the worldwide equine industry.

Although the horse is the natural host of the equine herpesviruses, a variety of animals and tissue culture systems can be used to propagate the viruses. Experimental animals for EHV-1 include Syrian hamsters, baby hamsters, chick embryos, baby and adult mice, and kittens. Primary tissue culture systems used to propagate EHV-1 include cells from a variety of equine tissues such as fetal lung, dermis, spleen and kidney, as well as cells from domestic cats, dogs, hamsters, rabbits, mice, sheep and swine. In the laboratory,

permanent tissue culture systems commonly used to cultivate EHV-1 include HeLa, Vero, CV-1, rabbit kidney (RK), mouse L-M and equine Edmin337 cells.

Natural respiratory EHV-1 infection of the horse only results in a short-lived humoral response and does not confer long-term protection against subsequent infection. A number of vaccines have been developed to combat EHV-1 infections, among them inactivated vaccines which mostly contain both EHV-1 and EHV-4 (Pneumabort K™, Resequin™, Prestige™ and Duvaxyn™), modified live vaccines (Rhinomune™ or Prevaccinol™) and subunit vaccines (Cavalon IR™). However, some EHV vaccines cause undesirable side effects, and most do not afford acceptable levels of protection. There is a need for safe and effective vaccines against EHV-1 infection.

The present invention is directed to mutant equine herpesvirus type-1 (EHV-1) isolates carrying one or more mutations in the immediate-early (IE) gene of the viral genome.

In particular, the present invention provides EHV-1 isolates carrying in the IE gene of the viral genome, at least one of the mutations listed in **Table 1**.

In a preferred embodiment, the present invention provides replication-competent EHV-1 isolates carrying one or more mutations in the IE gene.

In particular, the mutation in the IE gene does not significantly interfere with the structure and/or function of any of the four domains of the IE protein, TAD (aa 1-89), SRT (aa 181-220), DBD (aa 422-597) and NLS (aa 963-970), which are essential for viral replication.

In this embodiment, the present invention provides replication-competent EHV-1 isolates which carry a mutation in the IE gene that involves a substitution of an amino acid residue within TAD, SRT, DBD or NLS. Preferably, the substitution involves an exchange of amino acids within an exchange group, i.e., amino acids that resemble each other with respect to their overall impact on protein structure. More preferably, the substitution is one of D24N, D20N, F15D, L12E or E34Q.

Particularly preferred mutations include insertions or deletions of one or more, preferably, at least three, more preferably at least five, amino acid residues within amino acid 90-180, 221-421, 598-962, or 971-1487. Most preferred mutations include d644/824 (deletion of aa 644-824), n1029 (nonsense mutation at 1029 and deletion of aa 1030-1487), n1411 (nonsense mutation at 1411 and deletion of aa 1411-1487), in628 (insertion at 1411) and in1411 (insertion at 1411).

In a preferred embodiment, the present invention provides replication-competent EHV-1 isolates which carry one or more mutations in the IE gene and which have reduced virulence or no virulence, also referred herein as "non-pathogenic" EHV-1 isolates.

Non-pathogenic, replication competent EHV-1 isolates can be of an EHV-1 strain such as KyA, KyD, Ab4, Ab1, RacL11, RacH and RacM wherein one or more mutations have been introduced into the IE gene of the viral genome. Preferred mutant EHV-1 isolates are KyA mutant isolates.

The present invention is further directed to immunogenic compositions which include one or more of the non-

pathogenic, replication-competent mutant EHV-1 isolates described herein.

The present invention further provides methods of stimulating an immune response against EHV-1 in a horse subject by administering to the horse an immunogenic composition of the present invention.

Still further the present invention is directed to live attenuated vaccine compositions which include one or more of the non-pathogenic, replication-competent mutant EHV-1 isolates described herein.

Still even further, the present invention provides methods of treating EHV-1 infections in a horse by administering to the horse subject, a therapeutically effective amount of a non-pathogenic, replication-competent mutant EHV-1 isolate of the present invention.

Yet even further, the present invention provides methods for determining the pathogenicity of an EHV-1 strain present in a subject previously vaccinated with a non-pathogenic EHV-1 isolate of the present invention. The determination is achieved by carrying out assays which distinguish the wild type EHV-1 and the non-pathogenic EHV-1 isolate previously administered to the subject.

Figure 1 depicts the structure of the EHV-1 genome and location of the immediate-early gene. A schematic of the EHV-1 genome is shown at the top. The lower portion of the figure depicts the functional domains of the IE protein (1,487 amino acids): the transactivation domain (TAD, aa 3-89), a serine rich tract (SRT, aa 181-220), the DNA-binding domain

(DBD, aa 422-597), nuclear localization signal (NLS, aa 963-970).

Figure 2 depicts the recombination system to generate mutations in the IE gene. *Left panel*: Construct pIECassette contains the 5′ portion of the IE gene. Unique *Nco*I and *Nae*I restriction sites facilitate the cloning of sequences encoding mutagenized TADs spanning amino acids 3-89. *Right panel:* Construct pBR322IE contains the entire IE ORF. *Eco*RV/*Bam*HI fragments from various pIECassette constructs containing mutagenized TADs were easily cloned into pBR322IE to reconstitute an ORF that encodes a mutant form of the IE protein. Alternatively, various domains were swapped, replacing the wild-type sequences with various mutagenized sequences. This vector was used to generate recombinant viruses. Recombination plasmids were transfected into IE13.1 cells which were superinfected with KyAΔIE at 24 h post-transfection. Supernatants were screened on RK-13 cells for viruses that were able to propagate in the absence of complementing IE protein.

Figure 3 depicts the Western analyses of EHV-1 mutants. *Panel A*, infected-cell extracts (ICE) derived from RK-13 cells infected with EHV-1 KyA (Lane1), KyAd644/824 (Lane 2), KyAn1411 (Lane 3), or KyAIn1411 (Lane 4) at an MOI of 10. ICE were subjected to SDS-PAGE, and the proteins were blotted to nitrocellulose and were stained with the polyclonal anti-IE peptide antibody. The 200-kDa band representing the IE protein was clearly detected in Lanes 1, 3, and 4. The 175-kDa band derived from KyAd644/824-infected cells is shown in Lane 2. *Panel B*, Lane 1 shows the 200-kDa IE protein band detected in nuclear extracts of RK-13 cells infected with EHV-

1 KyA. Lane 2 shows the 138 kDa IE protein band detected in nuclear extracts of RK-13 cells infected with KyAn1029.

Figure 4 depicts the growth analyses of selected IE mutant viruses. RK-13 cells were infected with either wild-type EHV-1 KyA or selected EHV-1 IE mutant viruses at an MOI of 1 and incubated for 1 h at 37°C in 5% CO_2 to permit virus attachment. After attachment, the cells were washed three times with Eagle's without FBS to remove unattached virus and were incubated at 37°C in 5% CO_2. At the times indicated after the attachment period, virus titers in the culture supernatant were determined by plaque assay using RK-13 cells.

Figure 5A-5C depict the analysis of the CTL activity of the lymphocytes isolated from CBA mice immunized with wild type EHV-1 or EHV-1 containing mutations in the IE gene.

One aspect of the present invention is directed to novel mutant equine herpesvirus type-1 (EHV-1) isolates carrying one or more mutations in the immediate-early (IE) gene of the viral genome.

EHV-1 has a linear, double-stranded DNA genome, characterized by short and long unique sequences (U_S and U_L respectively), and inverted repeats which flank the unique short sequences. The entire genome of EHV-1, strain Ab4, has been sequenced and shown to be 150,223 bp in size and contain 80 potential open reading frames (ORFs).

The IE gene is the sole immediate-early gene of EHV-1 and is present in both inverted repeats of the viral genome. The open reading frame (ORF) of the IE gene (**SEQ ID NO: 1**) is transcribed to a 6.0-kb spliced mRNA that gives rise to both structurally and antigenically-related protein species

(Caughman et al. Virology 163:563-571, 1988). The predominant IE protein species of 1,487 amino acids (**SEQ ID NO: 2**) is comprised of discrete, functional domains (**Figure 1**). A potent transcriptional activation domain (TAD) maps within the first 89 amino acids. A serine rich tract (SRT; aa 181-220) may contain a site(s) for phosphorylation. The DNA-binding function lies within amino acid residues 422-597. Amino acids spanning 963-970 (NLS) are necessary for nuclear localization of the protein.

The IE protein is essential for viral growth in cell culture and is required for both early and late gene expression during the course of a productive infection (Smith et al. J. Virol. 66:936-945, 1992). Following IE polypeptide synthesis, approximately 45 early transcripts can be detected. Three of these early proteins serve as regulatory proteins and are designated EICP22, EICP27 and EICP0. Early gene expression is followed by the production of approximately 29 late transcripts and viral replication, after which mature virions are generated.

The term "mutation" as used herein includes substitution, deletion or insertion of one or more base pairs in the IE coding sequence which results in a substitution, deletion or insertion of one or more amino acid residues in the IE protein.

According to the present invention, EHV-1 isolates carrying mutations in the IE gene can be generated by a recombination system provided herein. In accordance with such system, mutations in the IE gene can be generated by employing any of the myriad recombinant cloning techniques, e.g., those described in *Current Protocols in Molecular Cloning* (Ausubel et al., John Wiley & Sons, New York). A mutant IE nucleotide

sequence carrying one or more mutations is then placed on a recombination vector appropriate for transfection and transfected into an appropriate host cell, e.g., RK-13 cells. Transfected host cells are then infected with a null EHV-1 virus devoid of the IE gene. Mutant viruses are thus generated by homologous recombination between the genome of the null virus and the recombination vector containing the mutant IE gene. Null EHV-1 viruses for use in this recombination system can be generated using any EHV-1 strain, e.g., KyA, KyD, Ab4, Ab1, RacL11, RacH and RacM. A deposit of the EHV-1 KyA strain was made with the American Tissue Type Culture, 10801 University Blvd., Manassas, VA 20110-2209, on July 20, 2000 (ATCC deposit # PTA-2253). Null virus of strain KyA, i.e., KyAΔIE, has been generated as described by Garko-Buczynski et al. (Virology 248: 83-94, 1998). By way of this recombination system, mutant EHV-1 isolates carrying identical or different mutations in the two copies of the IE gene can be generated. Preferably, the mutant EHV-1 isolates of the present invention carry identical mutation(s) in both copies of the IE gene in the viral genome.

One embodiment of the present invention provides EHV-1 isolates carrying in the IE gene of the viral genome, at least one of the mutations listed in Table 1.

Table 1

Deletion Mutations	Nature of Mutation	Domain Affected
ΔSRT1	aa 181-250 deleted	Deletion of SRT
ΔSRT2	aa 88-243 deleted	Deletion of SRT
d178/627	aa 178 to 627 deleted	Deletion of SRT and DBD
d552/897	aa 552 to 897 deleted	Deletion of part of DBD

d644/824	aa 644 to 824 deleted	
Nonsense Mutations		
n627	aa 628-1487 deleted	Deletion of NLS and C-term
n951	aa 952-1487 deleted	Deletion of NLS and C-term
n1029	aa 1030 -1487 deleted	Deletion of C-term
n1411	aa 1412 - 1487 deleted	Deletion of C-term
Insertion Mutations		
in628	insertion at aa 628	
in1411	insertion at aa 1411	
Point Mutations: Amino Acid Substitutions		
D24N	Asp 24 to Asn	Point mutation in TAD
D20N	Asp 20 to Asn	Point mutation in TAD
F15D	Phe 15to Asp	Point mutation in TAD
L12P	Leu 12 to Pro	Point mutation in TAD
L12E	Leu 12 to Glu	Point mutation in TAD
E34Q	Glu 34 to Gln	Point mutation in TAD

In a preferred embodiment, the present invention provides mutant EHV-1 isolates that are replication-competent.

The term "replication competence" as used herein refers to the ability of a viral isolate to propagate in a host cell in the absence of a complementing IE protein expressed in *trans*.

According to the present invention, the replication competence of a mutant viral isolate can be determined by a number of assays, e.g., a plaque assay using non-complementing cells (cells that do not express IE protein). In this assay, cells of a monolayer are infected with a mutant isolate of interest and are subsequently overlaid with 2% agarose mixed in a 1:1 ratio with appropriate growth medium. Host cells which can be employed for this purpose include cells from a

variety of equine tissues such as fetal lung, dermis, spleen and kidney, as well as cells from domestic cats, dogs, hamsters, rabbits, mice, sheep and swine. Typically, cells commonly used to cultivate EHV-1 in tissue culture are used, including HeLa, Vero, CV-1, rabbit kidney (RK), mouse L-M and equine Edmin337 cells. Preferably, RK-13 cells are used in the assay.

A mutant viral isolate is "replication competent" if such mutant isolate can form plaques on the non-complementing cells, even if the plaques may be of a smaller size than those formed by wild type EHV-1. Replication-incompetent cells can only form plaques in complementing cells where the IE protein is expressed in trans e.g., IE13.1 cells.

According to the present invention, four domains of the IE protein, TAD (aa 1-89), SRT (aa 181-220), DBD (aa 422-597) and NLS (aa 963-970), are essential for viral replication. Thus, mutations in the IE gene that significantly interfere with the structure and/or function of these four domains likely produce replication incompetent viral isolates. For example, a deletion of five or more contiguous amino acid residues within any of these four domains of the IE protein is likely disruptive to the function of such domain, and the resulting mutant virus is likely to be replication-incompetent. However, substitution of one or more amino acid residues to residues similar in size and/or hydrophobicity can be less disruptive to the structure and/or function of the respective domain and thus, the resulting mutant virus can still be replication-competent.

Accordingly, one embodiment of the present invention provides replication-competent EHV-1 isolates harboring at least one mutation in the IE gene, wherein the mutation is a

substitution at a residue anywhere within TAD (aa 1-89), SRT (aa 181-220), DBD (aa 422-597) or NLS (aa 963-970) and wherein the mutation does not substantially disrupt the function of the IE protein in viral replication.

Although not limited to any specific formula, a preferred substitution according to the present invention can be between amino acids within an exchange group, i.e., amino acids that resemble each other with respect to their overall impact on protein structures. For example, aromatics Phe, Tyr and Typ form an exchange group; the positively charged residues Lys, Arg and His form an exchange group; the large aliphatic non-polar residues Val, Leu and Ile form an exchange group which also contains the slightly polar Met and Cys. All small residues Ser, Thr, Asp, Asn, Gly, Ala, as well as Glu, Gln and Pro are also within an exchange group.

More preferably, the substitution is one of D24N, D20N, F15D, L12E or E34Q. Even more preferably, the mutant EHV-1 isolates carrying one or more of these subtitutions are of a strain selected from KyA, KyD, Ab1, Ab4, RacL11, RacM or RacH. Particularly preferred mutant EHV-1 isolates are KyAD24N, KyAD20N, KyAF15D, KyAL12E and KyAE34Q.

In another preferred embodiment of the present invention, the replication-competent mutant EHV-1 isolates harbor a mutation that is localized outside of any of the four domains described above and does not cause any substitution, deletion or insertion within any of the TAD (aa 1-89), SRT (aa 181-220), DBD (aa 422-597) and NLS (aa 963-970) domains.

Particularly preferred mutations include insertions or deletions of one or more, preferably, at least three, more preferably at least five, amino acid residues within the region of amino acid 90-180, 221-421, 598-962, or 971-1487.

Most preferred mutations include d644/824 (deletion of aa 644-824), n1029 (nonsense mutation at 1029 and deletion of aa 1030-1487), n1411 (non-sense mutation at 1411, and thus deletion of aa 1411-1487), in628 (insertion at 1411) and in1411 (insertion at 1411). The mutant EHV-1 isolates carrying one or more of these mutations are preferably of a strain selected from KyA, KyD, Ab1, Ab4, RacL11, RacM or RacH, and more preferably, KyA.

In a preferred embodiment, the present invention provides replication-competent mutant EHV-1 isolates that have reduced virulence or no virulence, also referred herein as "non-pathogenic" or "attenuated" EHV-1 isolates.

The term "virulence" or "pathogenicity" as used herein refers to the capacity of a strain of EHV-1 to induce EHV-1-related diseases in horses, e.g., infection in the respiratory tracts, spontaneous abortions as well as neurological diseases. Infections caused by pathogenic EHV-1 are typically characterized by fever, profuse nasal discharge and congestion of the nasal mucosa. Accordingly, a "non-pathogenic" or "attenuated" strain of EHV-1, or an EHV-1 strain with "reduced virulence" as used herein is a strain having a substantially reduced capacity, as compared to a pathogenic EHV-1, in inducing the development of EHV-related clinical diseases.

According to the present invention, the pathogenicity of an EHV-1 isolate can be conveniently determined in mouse models. Clinical signs of EHV-1 infection in mice include, e.g., ruffled fur, loss of body weight, labored breathing, lethargy and huddling, as described by, e.g., Colle et al. Virus Res. 43: 111-124 (1996) and Zhang et al. Virus Res. 56: 11-24 (1998). In addition, the degree of

infection can also be assessed by isolating the viruses from the lungs of infected mice, plating the viruses on RK-13 cell monolayers and determining the number of plaques formed, as described in the Examples that follow.

The non-pathogenic mutant EHV-1 isolates of the present invention can be generated by introducing a mutant IE gene into null viruses of a non-pathogenic EHV-1 strain via the recombinant system described herein. Any of those naturally non-pathogenic EHV-1 strains or EHV-1 strains that are made non-pathogenic by laboratory procedures can be used for generating null-viruses, e.g., KyA, KyD, Ab4, Ab1, RacL11, RacH and RacM. Null viruses of any of these non-pathogenic EHV-1 strains can be generated, e.g., by following the procedure described by Garko-Buczynski et al. (Virology 248: 83-94, 1998).

A preferred EHV-1 strain for use in generating the mutant isolates of the present invention is the EHV-1 KyA strain, a deposit of which was made with the American Tissue Type Culture, 10801 University Blvd., Manassas, VA 20110-2209, on July 20, 2000 (ATCC deposit # PTA-2253).

Accordingly, preferred non-pathogenic, replication competent mutant EHV-1 isolates of the present invention include KyA isolates carrying at least one mutation in the IE gene, wherein the mutation is a substitution at a residue anywhere within TAD (aa 1-89), SRT (aa 181-220), DBD (aa 422-597) or NLS (aa 963-970) and wherein the mutation does not substantially disrupt the function of the IE protein in viral replication. Examples of such mutant EHV-1 isolates are KyAD24N, KyAD20N, KyAF15D, KyAL12E and KyAE34Q.

Other preferred non-pathogenic, replication competent mutant EHV-1 isolates of the present invention

include KyA isolates carrying at least one mutation in the IE gene, wherein the mutation is localized outside of any of the four domains described above and does not cause any substitution, deletion or insertion within any of the TAD (aa 1-89), SRT (aa 181-220), DBD (aa 422-597) and NLS (aa 963-970) domains. Examples of such mutant EHV-1 isolates are KyAd644/824, KyAn1029, KyAn1411, KyAin628 and KyAin1411.

In a further aspect of the invention, the non-pathogenic, replication-competent mutant EHV-1 isolates of the present invention as described hereinabove are included in immunogenic compositions.

By "immunogenic" is meant the capacity of an EHV-1 isolate in provoking a immune response in a horse subject, either a cellular immune response mediated primarily by cytotoxic T-cells, or a humoral immune response mediated primarily by helper T-cells which in turn activate B-cells leading to antibody production.

The immunogenic compositions of the present invention include at least one, i.e., one or more of the non-pathogenic replication competent mutant EHV-1 isolates described hereinabove. Preferred mutant EHV-1 isolates to be included in the immunogenic compositions include, e.g., an EHV-1 isolate harboring one or more of the mutations D24N, D20N, F15D, L12E or E34Q in the IE gene, or an EHV-1 isolate harboring one or more mutations within aa 90-180, 221-421, 598-962 or 971-1487 of the IE protein. Preferably, the mutant EHV-1 isolates for use in an immunogenic composition of the present invention are of a non-pathogenic EHV-1 strain such as KyA, KyD, Ab4, Ab1, RacL11, RacH and RacM. Particularly preferred mutant EHV-1 isolates for use in an immunogenic

composition include KyAD24N, KyAD20N, KyAF15D, KyAL12E, KyAE34Q, KyAd644/824, KyAn1411 and KyAin1411.

The immunogenic compositions of the present invention can also include additional active ingredient such as other immunogenic compositions against EHV-1, e.g., those described in U.S. Patent 5,707,629 ("Immunogenic composition against equine herpesvirus type 1") and U.S. Patent 5,795,578 ("Vaccine against equine herpesvirus type 1), or immunogenic compositions against EHV-4.

In addition, the immunogenic compositions of the present invention can include one or more pharmaceutically-acceptable carriers.

As used herein "a pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, adjuvant, vaccine stabilizing agents, antibacterial and antifungal agents, isotonic agents such as sugar and sodium chloride, adsorption delaying agents, and the like. The use of such media gents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the immunogenic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The immunogenic compositions of the present invention can made in forms suitable for injectable use, e.g., in the form of sterile aqueous solutions or dispersions, or can be made in lyophilized forms using vacuum-drying and freeze-drying techniques. Lyophilized vaccine compositions are typically maintained at about 4°C, and can be reconstituted in a stabilizing solution, e.g., saline or and

HEPES, with or without adjuvant. In all cases the form of the immunogenic compositions must be sterile.

The immunogenic compositions of the present invention can be administered to a horse to induce an immune response against EHV-1. Accordingly, another embodiment of the present invention provides methods of stimulating an immune response against EHV-1 in a horse subject by administering an effective amount of any one of the above-described immunogenic compositions of the present invention.

The amount of an immunogenic composition to be adminisitered to be "effective" in inducing an immune response may depend on the immunogenicity of the particular EHV-1 isolate used in the immunogenic composition. According to the present invention, the immunogenicity of an EHV-1 isolate, i.e., the type and extent of an immune response induced by a viral isolate can be conveniently assessed in mice, which are generally accepted as models representative of horse subjects. A variety of techniques may be used for analyzing the immune responses induced in mice by a viral isolate. For example, one skilled in the art can determine whether a viral isolate induces a cell-mediated immune response by, e.g., detecting the presence of responding CTLs in the mouse spleen or other lymphoid tissues. One skilled in the art can also readily determine whether a viral isolate stimulates a humoral immune response by, e.g., detecting the neutralizing titer of EHV-1 specific antibody in the serum or the presence of antibody secreting cells in the serum. These techniques are well described in the art, e.g., Coligan et al. Current Protocols in Immunology, John Wiley & Sons Inc. (1994).

For inducing an immune response, an immunogenic composition of the present invention can be administered to a

horse subject via intravenous, intraperitoneal, intramuscular, or intramucosal (e.g. nasal or respiratory spray or injection) routes, or by other forms of parenteral administration. An immunogenic composition can also be administered via an implant or orally.

Another embodiment of the present invention is directed to live attenuated vaccine compositions.

More specifically, the vaccine compositions of the present invention include one or more of the non-pathogenic, replication-competent mutant EHV-1 isolates as described hereinabove.

The infection of a cell or cells by a pathogenic strain of EHV-1 leads to the production of pathogenic virions in the infected subjects and the EHV-1 related diseases. In contrast, a non-pathogenic EHV-1 strain of the present invention generally replicates to an extent to sufficient to protect the subject against challenge by a virulent or pathogenic EHV-1 strain.

The term "vaccine" as used herein refers to a composition which prevents or reduces the risk of infection or which ameliorates the symptoms of infection. The protective effects of a vaccine composition against a pathogen are normally achieved by stimulating an immune response in the subject which may involve either or both of cell-mediated or humoral immune response. The strength and duration of the immune responses induced by an EHV-1 isolate can be taken into consideration in determining the amount of such isolate that should be included in a vaccine composition, as well as the vaccination schedules. Generally speaking, abolished or reduced incidences of EHV-1 infection, amelioration of the symptoms, or accelerated elimination of the viruses from the

infected subjects are indicative of the protective effects of a vaccine composition.

Preferred non-pathogenic, replication competent mutant EHV-1 isolates which can be used in a vaccine composition include, e.g., an EHV-1 isolate harboring one or more of D24N, D20N, F15D, L12E or E34Q in the IE gene. Preferably, the mutant EHV-1 isolate for use in a vaccine composition is of a non-pathogenic EHV-1 strain such as KyA, KyD, Ab4, Ab1, RacL11, RacH and RacM. Particularly preferred EHV-1 isolates for use in a vaccine composition include KyAD24N, KyAD20N, KyAF15D, KyAL12E and KyAE34Q.

Other preferred non-pathogenic, replication competent mutant EHV-1 isolates which can be used in a vaccine composition include an EHV-1 isolate harboring one or more mutations within aa 90-180, 221-421, 598-962, or 971-1487 of the IE protein, for example, d644-824, n1411 and in1411. Preferably, the mutant EHV-1 isolate for use in a vaccine composition is of a non-pathogenic EHV-1 strain such as KyA, KyD, Ab4, Ab1, RacL11, RacH and RacM. Particularly preferred EHV-1 isolates for use in a vaccine composition include KyAd644/824, KyAn1411 and KyAin1411.

The vaccine compositions of the present invention can also include additional active ingredient such as other immunogenic compositions against EHV-1, e.g., those described in U.S. Patent 5,707,629 ("Immunogenic composition against equine herpesvirus type 1") and U.S. Patent 5,795,578 ("Vaccine against equine herpesvirus type 1), or immunogenic compositions against EHV-4.

Furthermore, the vaccine compositions of the present invention can include one or more pharmaceutically-acceptable carriers as described hereinabove.

The vaccine compositions of the present invention can made in forms suitable for injectable use, e.g., in the form of sterile aqueous solutions or dispersions, or can be made in lyophilized forms using vacuum-drying and freeze-drying techniques. Lyophilized vaccine compositions are typically maintained at about 4°C, and can be reconstituted in a stabilizing solution, e.g., saline or and HEPES, with or without adjuvant. In all cases the form of the vaccine compositions must be sterile.

In another embodiment of the present invention, the above vaccine compositions of the present invention are used in treating EHV-1 infections. Accordingly, the present invention provides methods of treating EHV-1 infections in a horse by administering to the horse subject, a therapeutically effective amount of a non-pathogenic EHV-1 isolate of the present invention.

By "treating" is meant preventing or reducing the risk of infection by a pathogenic strain of EHV-1, ameliorating the symptoms of an EHV-1 infection, or accelerating the recovery from an EHV-1 infection.

The amount of a non-pathogenic EHV-1 isolate that is therapeutically effective may depend on the nature of the isolate, the condition of the horse and/or the degree of infection, and can be determined by a veterinary physician.

In practicing the present methods, a vaccine composition of the present invention can be administered to a horse subject via intravenous, intraperitoneal, intramuscular, or intramucosal (e.g. nasal or respiratory spray or injection) routes, or by other forms of parenteral administration. A vaccine composition can also be administered via an implant or orally. Boosting regiments may be required and the dosage

regimen can be adjusted to provide optimal immunization. The vaccination of a mare prior to breeding and again during her pregnancy may prevent abortions caused by EHV-1. Other horses can be vaccinated, for example, about once a year. Foals can be vaccinated shortly after birth.

Non-pathogenic, replication competent mutant EHV-1 isolates of the present invention can also have immune protective effects against infections caused by equine herpesvirus type 4 (EHV-4). Thus, the use of the mutant EHV-1 isolates of the present invention in immunogenic or vaccine compositions for preventing or inhibiting EHV-4 infections is also contemplated by the present invention.

The vaccine compositions of the present invention have the additional feature that the non-pathogenic EHV-1 isolate included therein is generally discernable from wild type EHV-1 strains in terms of the composition and size of the IE protein expressed, or the sensitivity of growth to temperature. This feature is useful, e.g., in determining whether a subject tested positive for EHV-1 in certain laboratory tests carries a pathogenic EHV-1 or a previously inoculated non-pathogenic EHV-1.

Accordingly, in another embodiment, the present invention provides methods of determining the pathogenicity of an EHV-1 strain present in a subject previously vaccinated with a non-pathogenic EHV-1 isolate of the present invention.

As the methods are premised on a distinction between the wild type EHV-1 and the non-pathogenic EHV-1 isolate previously administered to the subject, the choice of assays for making the distinction depends on the nature of the mutation of the non-pathogenic EHV-1 isolate.

When the non-pathogenic viral isolate previously administered to the subject contains a deletion in the IE gene, EHV-1 having d644/824, n1029 or n1411, assays based on detection of IE proteins or antibodies in serum against IE proteins can be employed. For example, infected cells or tissues can be isolated from the subject. Intracellular extracts can be made from such cells or tissues and can be subjected to, e.g., Western Blot analysis, as described in the Examples hereinbelow. The observation of an IE protein of a lower Mw is indicative of the non-pathogenicity of the EHV-1 strain present in the subject. Alternatively, an antibody specific for the deleted portion of amino acid residues can be used in Western Blot, and absence of an IE protein band is also indicative of the non-pathogenicity of the EHV-1 strain present in the subject. Additionally, the absence of antibodies in the serum against the deleted or truncated portion of the IE protein, may also be an indication of the non-pathogenicity of the virus in the subject. The presence or absence of antibodies in the serum of the subject can be determined by using a peptide corresponding to the deleted portion in an appropriate immunoassay, e.g., ELISPOT.

When the non-pathogenic viral isolate previously used in vaccination has a different sensitivity to temperature as compared to wild type EHV-1, temperature shift assays can also be used, as described in the Examples hereinbelow. For example, such assays can be applied in distinguishing wild type EHV-1 from, e.g., EHV isolates having d644/824, n1029, n1411, in1411, D24N, L12E, F15D or E34Q, and in particular, n1029 and n1411. Unlike wide type KyA, KyAn1411 and KyA1029 fail to grow at 39 C. KyA carrying d644/824, in1411, D24N,

L12E, F15D or E34Q has a reduced capacity to grow as compared to wild type KyA.

The determination of the pathogenicity can also be achieved by using nucleic acid-based assays to screen for mutations in the IE gene of the viruses isolated from the subject. These assays include Southern or Northern blot analysis, PCR, and sequencing.

A mutation in the IE gene may result in a reduced expression of another EHV-1 protein, e.g., EICP0 or gD. For example, n1029 causes significant reduction in the mRNA levels of both EICP0 and gD. In this case, detection of the reduced expression of such other proteins, either at the mRNA level or the protein level is indicative of the non-pathogenicity of the virus in the subject.

Any variations of the foregoing assays are also encompassed by the present invention.

The present invention is further illustrated by the following examples.

We claim:

1. An EHV-1 isolate comprising a mutation selected from Table 1.

2. The EHV-1 isolate of claim 1, wherein said EHV-1 isolate is of a strain selected from KyA, KyD, Ab1, Ab4, RacL11, RacM or RacH.

3. An EHV-1 isolate selected from the group consisting of KyAΔSRT1, KyAΔSRT2, KyAd178/627, KyA552/897, KyA644/824, KyAn627, KyAn951, KyAn1029, KyAn1411, KyAin628, KyAin1411, KyAD20N, KyAD24N, KyAL12P, KynL12E, KyAF15D and KyAE34Q.

4. A replication-competent EHV-1 isolate comprising a mutation in the IE gene.

5. The EHV-1 isolate of claim 4, wherein said mutation is a substitution of an amino acid residue within aa 1-89, 181-220, 422-597 or 963-970.

6. The EHV-1 isolate of claim 5, wherein said substitution is selected from D20N, D24N, L12E, F15D or E34Q.

7. The EHV-1 isolate of claim 4, wherein said mutation is a deletion or insertion of at least about three amino acid residues within aa 90-180, 221-421, 598-962 or 963-1487.

8. The EHV-1 isolate of claim 7, wherein said mutation is selected from d644/824, n627, n1029, n1411, in628 or in1411.

9. The EHV-1 isolate of claim 4, wherein said EHV-1 isolate is non-pathogenic.

10. The EHV-1 isolate of claim 9, wherein said EHV-1 isolate is of a strain selected from KyA, KyD, Ab1, Ab4, RacL11, RacM or RacH.

11. The EHV-1 isolate of claim 10, wherein the mutation in the IE gene of said EHV-1 isolate is a substitution of an amino acid residue within aa 1-89, 181-220, 422-597 or 963-970.

12. The EHV-1 isolate of claim 11, wherein said mutation is selected from D20N, D24N, L12E, F15D or E34Q.

13. The EHV-1 isolate of claim 12, selected from KyAD20N, KyAD24N, KyAL12E, KyAF15D or KyAE34Q.

14. The EHV-1 isolate of claim 10, wherein the mutation in the IE gene of said EHV-1 isolate is a deletion or insertion of at least about three amino acid residues within aa 90-180, 221-421, 598-962 or 963-1487.

15. The EHV-1 isolate of claim 14, wherein said isolate is selected from d644/824, n627, n1029, n1411, in628 or in1411.

16. The EHV-1 isolate of claim 15 selected from KyAd644/824, KyAn627, KyAn1029, KyAn1411, KyAin628 or KyAin1411.

17. A immunogenic composition comprising a pharmaceutical-acceptable carrier and a nonpathogenic, replication-competent EHV-1 isolate, wherein said EHV-1 isolate comprises a mutation in the IE gene.

18. The immunogenic composition of claim 17, wherein said EHV-1 isolate is of a non-pathogenic strain selected from KyA, KyD, Ab1, Ab4, RacL11, RacM or RacH.

19. The immunogenic composition of claim 18, wherein the mutation in the IE gene of said isolate is a substitution of an amino acid residue within aa 1-89, 181-220, 422-597 or 963-970.

20. The immunogenic composition of claim 19, wherein said mutation is one of D20N, D24N, L12E, F15D or E34Q.

21. The immunogenic composition of claim 20, wherein said EHV-1 isolate is selected from KyAD20N, KyAD24N, KynL12E, KyAF15D or KyAE34Q.

22. The immunogenic composition of claim 18, wherein the mutation in the IE gene of said isolate is a deletion or insertion of at least about three

amino acid residues within aa 90-180, 221-421, 598-962 or 963-1487.

23. The immunogenic composition of claim 22, wherein said mutation is selected from d644/824, n627, n1029, n1411, in628 or in1411.

24. The immunogenic composition of claim 23, wherein said isolate is selected from KyAd644/824, KyAn627, KyAn1029, KyAn1411, KyAin628 or KyAin1411.

25. A method of stimulating an immune response against EHV-1 in a horse subject, comprising administering to said subject an effective amount of a nonpathogenic, replication-competent EHV-1 isolate, wherein said EHV-1 isolate comprises a mutation in the IE gene.

26. The method of claim 25, wherein said EHV-1 isolate is of a non-pathogenic strain selected from KyA, KyD, Ab1, Ab4, RacL11, RacM or RacH.

27. The method of claim 26, wherein the mutation in the IE gene of said isolate is a substitution of an amino acid residue within aa 1-89, 181-220, 422-597 or 963-970.

28. The method of claim 27, wherein said mutation is one of D20N, D24N, L12E, F15D or E34Q.

29. The method of claim 28, wherein said EHV-1 isolate is selected from KyAD20N, KyAD24N, KynL12E, KyAF15D or KyAE34Q.

30. The method of claim 26, wherein the mutation in the IE gene of said isolate is a deletion or insertion of at least about three amino acid residues within aa 90-180, 221-421, 598-962 or 963-1487.

31. The method of claim 30, wherein said mutation is selected from d644/824, n627, n1029, n1411, in628 or in1411.

32. The method of claim 31, wherein said isolate is selected from KyAd644/824, KyAn627, KyAn1029, KyAn1411, KyAin628 or KyAin1411.

33. The method of claim 25, wherein said immune response is a cell-mediated immune response or a humoral immune response.

34. An vaccine composition comprising a pharmaceutical-acceptable carrier and a nonpathogenic, replication-competent EHV-1 isolate, wherein said EHV-1 isolate comprises a mutation in the IE gene.

35. The vaccine composition of claim 34, wherein said EHV-1 isolate is of a non-pathogenic strain selected from KyA, KyD, Ab1, Ab4, RacL11, RacM or RacH.

36. The vaccine composition of claim 35, wherein the mutation in the IE gene of said isolate is a substitution of an amino acid residue within aa 1-89, 181-220, 422-597 or 963-970.

37. The vaccine composition of claim 36, wherein said mutation is one of D20N, D24N, L12E, F15D or E34Q.

38. The vaccine composition of claim 37, wherein said EHV-1 isolate is selected from KyAD20N, KyAD24N, KynL12E, KyAF15D or KyAE34Q.

39. The vaccine composition of claim 35, wherein the mutation in the IE gene of said isolate is a deletion or insertion of at least about three amino acid residues within aa 90-180, 221-421, 598-962 or 963-1487.

40. The vaccine composition of claim 39, wherein said mutation is selected from d644/824, n627, n1029, n1411, in628 or in1411.

41. The vaccine composition of claim 40, wherein said isolate is selected from KyAd644/824, KyAn627, KyAn1029, KyAn1411, KyAin628 or KyAin1411.

42. A method of preventing or inhibiting an EHV-1 infection in a horse subject, comprising administering to said subject a therapeutically effective amount of a nonpathogenic, replication-

competent EHV-1 isolate, wherein said EHV-1 isolate comprises a mutation in the IE gene.

43. The method of claim 42, wherein said EHV-1 isolate is of a non-pathogenic strain selected from KyA, KyD, Ab1, Ab4, RacL11, RacM or RacH.

44. The method of claim 43, wherein the mutation in the IE gene of said isolate is a substitution of an amino acid residue within aa 1-89, 181-220, 422-597 or 963-970.

45. The method of claim 44, wherein said mutation is one of D20N, D24N, L12E, F15D or E34Q.

46. The method of claim 45, wherein said EHV-1 isolate is selected from KyAD20N, KyAD24N, KynL12E, KyAF15D or KyAE34Q.

47. The method of claim 43, wherein the mutation in the IE gene of said isolate is a deletion or insertion of at least about three amino acid residues within aa 90-180, 221-421, 598-962 or 963-1487.

48. The method of claim 47, wherein said mutation is selected from d644/824, n627, n1029, n1411, in628 or in1411.

49. The method of claim 48, wherein said isolate is selected from KyAd644/824, KyAn627, KyAn1029, KyAn1411, KyAin628 or KyAin1411.

50. A method of determining the non-pathogenicity of an EHV-1 virus present in a horse subject previously administered with a non-pathogenic EHV-1 isolate, wherein said EHV-1 isolate comprises a mutation in the IE gene, said method comprising isolating said virus from said subject, detecting the presence of the mutant IE protein of said non-pathogenic isolate and the absence of a wild type IE protein in said virus, thereby determining said virus as non-pathogenic.

51. The method of claim 50, wherein the mutant IE protein has a mobility on SDS PAGE different from that of a wild type IE protein.

52. The method of claim 51, wherein the mutant IE protein comprises a deletion or insertion of amino acid residues, and wherein said detection of the IE protein is based on an antibody specific for the deleted or inserted amino acid residues.

53. A method of determining the non-pathogenicity of an EHV-1 virus present in a horse subject previously administered with a non-pathogenic EHV-1 isolate, wherein said EHV-1 isolate comprises a deletion in the IE gene, said method comprising detecting the absence in the serum of said subject of an antibody specific for the deleted portion of the IE protein, thereby determining said virus as non-pathogenic.

54. A method of determining the non-pathogenicity of an EHV-1 virus present in a horse subject previously administered with a non-pathogenic EHV-1 isolate, wherein said EHV-1 isolate comprises a mutation in the IE gene, said method comprising isolating said virus from said subject, detecting the absence of the wild type IE nucleotide sequence and the presence of the mutant IE nucleotide sequence, thereby determining said virus as non-pathogenic.

55. A method of determining the non-pathogenicity of an EHV-1 virus present in a horse subject previously administered with a non-pathogenic EHV-1 isolate, wherein said EHV-1 isolate comprises a mutation in the IE gene, said method comprising isolating said virus from said subject, determining the temperature sensitivity of said virus as identical to that of said non-pathogenic EHV-1 isolate, thereby determining said virus as non-pathogenic.